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Embryonic Development Of Rainbow Trout (Salmo Irideus Gibb.) From Overripe Eggs

by

Lilyana Dimcheva-Grozdanova

The retention of eggs in the fish's body, following ovulation, leads to their getting overripe. A number of authors have drawn attention to the fact that overripe eggs may become the cause of breeding anomalies, such as a diminished fertilizing capacity, high death incidence among embryos, both during and after their embryonic development, the appearance of abnormalities in embryos, etc. 83*

The earliest experiments with overripe eggs of rainbow trout were carried out by Mrsik (1923). The author worked with 7, 13 and 21 days overripe eggs. His observations concerned the period after the hatching of the embryos. He established a great number of abnormalities in the development of the experimental fishes, such as shortening of the operculum,

*The figures in the right-hand margin indicate page numbers of the original (Tr.).

lack of eyes and abdominal fins, curving of the spine, etc. The fishes were also slow to grow in size.

Research on the influence of overripe eggs on the embryonic development of various types of fishes has also been carried out by a number of Soviet specialists.

In experiments with 7 and 14 days overripe salmon eggs (Salmo salar m.relictus), Privolnev and Kharchenko established that overripe eggs were less resistant to outside influence and that mortality among embryos, hatched from overripe eggs, was higher. Later Kharchenko (1949, 1957) carried out further experiments with overripe eggs. In 1949 he worked with 7, 14 and 16 days overripe salmon eggs and established their fertilizing capacity, mortality rate, etc. The experimental embryos were, in general, paler in colour. In 1957 Kharchenko carried out further experiments with Coregonus albula.In both cases 100 per cent of the overripe eggs were fertilized. According to Kharchenko, overripeness does not affect the eggs' capacity for fertilization, meaning that overripe eggs retain their fertilizing capacity for a long time.

Nussenbaum (1949) obtained a considerable number of deformed embryos from overripe eggs of Misgurnus fossilis. High temperature was found to have a particularly deleterious effect on the overripening of eggs.

Vernidub (1951a, 1951b) wrote that sturgeon eggs, if retained longer in the sexual glands or in the abdominal cavity, gradually lose their fertilizing capacity. Kazanskii (1951) 84

Vernidub and Kisseleva (1953) also established that in the case of the sturgeon varieties, overripe eggs may lead to atypical developments, the occurrence of abnormalities in the embryonic structure and the death of eggs at an early stage of their development.

Interesting experiments were carried out by a collective of Chinese specialists (Thou-Su et al., 1960) on the embryonic development of carp and gold-fish (Carassius auratus), hatched from overripe egs. They established that overripe eggs had a sharply reduced level of fertilization. The authors also found out that the time for the normal fertilization of those fishes was limited, after which anomalies in the embryogenesis began to occur.

Overripeness does not affect in an equal way eggs of different sizes of the same fish variety. Interesting experiments in this respect were carried out by Chen-Chen (1961), who established that larger overripe carp or Coregonus albula eggs produced embryos with lower viability, whereas the embryos produced by smaller overripe eggs were very much like the ones in the control group.

In an article on the effect of overripeness on the embryonic development of fishes, Korovina (1963) noted that for the different fish varieties the duration of overripness was different and was determined by the specific ecological conditions.

It is evident from the above review of publications, that it was only Mrsik (1923) who carried out experiments with

overripe eggs of rainbow trout. The main objective of his study was to investigate the influence of overripeness on the embryos' sex. Along with this, he described the changes observed in the experimental fishes after being hatched. The author did not discuss the effect of overripenss on the development of the embryos prior to their hatching.

The object of the present article is to examine the embryonic development of rainbow trout (Salmo irideus Gibb.) produced from overripe eggs, the fertilizing capacity of the eggs, the fishes' growth rate and deviations from normal development.

Material and Methods

The experiments were carried out in 1964 and 1965 with eggs of rainbow trout, obtained from the State Fish Farm in Samokov. The eggs, both fertilized and unfertilized, were carried over to Sofia in petri dishes insulated with damp moss and ice cubes. The sperm was carried in dry test-tubes in a thermos filled with ice. The hatching was done in Californian-type incubators. The first experiments in May and June of 1964 were carried out with 6 female fishes. On May 3 we squeezed part of the eggs from three fishes, which were fertilized by sperm from two male fishes. The mothers were separated in a side pool with an average water temperature of 7^0 to 10^0C in the period of May 3 to May 10. A week later the mothers were caught again and were found to have spawned despite the unfavourable conditions. We had to fertilize the eggs of three other fishes,

which were placed in another pool without running water. The average water temperature for the period May 10 to May 18 was 6^0 to 9^0C. From the same female fishes (No.4, 5 and 6) a second lot of eggs was fertilized 4 days later. After 4 days, eggs could be squeezed from only fish No.6. Generally speaking, during the 1964 experiments, embryos were obtained from normal eggs, 4 days and 8 days overripe eggs. Mortality rates during the incubation period, the percentage of unfertilized eggs, and the time (measured in days and degree-days) elapsed until the beginning of hatching, were recorded. The data has been presented in the respective tables.[1] 85

In 1965 (from April 20 until the end of June) we carried out new experiments with eggs obtained from five mothers. We marked them by cutting a small piece from their abdominal, pectoral, anal or caudal fins. This time, from the same fishes

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[1]The same eggs, (normal, 4 and 8 days overripe) were used by Dr.G.Russev in bio-chemical tests. Dr.Russev studied membrane permeability by using the method of penetration of radioactive isotopes. He also studied the denaturation of proteins by the sorption method. He found that in the case of normal eggs, J131-albumin does not penetrate, whereas in the case of 8 days overripe eggs, it penetrates into all fish-eggs. Sorption was also found to increase with the increase in ripeness. More detailed results will be published by the author.

we managed to fertilize normally ripe eggs and 7 and 14 overripe eggs. The experimental fishes were kept in a pool with water temperature of $4^0$to 9^0C after the first removal of eggs, and $4^0$to 8^0C after the second one. The fertilization was done with sperm from 3 male fishes. Samples of the developing embryos were regularly fixed in a 10% formalin solution with the addition of iced vinegar acid, as well as in the Bouen fixative. The first 24 hours after fertilization, samples were fixed every half hour, and 2 to 3 times a day, thereafter. The water temperature in the incubator was taken 3 times a day. The oxygen content of the water varied between 9.9 to 10.5 mg/l of water. Eggs produced on the fish farm at the same time when the experimental eggs were removed from the fishes, were used as controls. A series of microscopic preparations, tinted with Karachi chematoxiline was prepared from the embryos fixed in the 10% formalin solution. After undergoing dehydration with carbol-xylol, the embryos were embalmed in Canadian balsam.

Results

The stages of embryonic development of rainbow trout from the moment of fertilization to the beginning of hatching have been established using living and fixed material, as well as the available information on other representatives of the Salmonidae Family (Leshchinskaya, 1953, Dissler, 1954 and 1957, Vassilev, 1959, Frolenko, 1959)and are based on the theoretical works of the well-known Soviet ichthyologists Kryzhanovskii (1949) and Vasnetsov (1953).

In order to study the changes in the embryonic development of overripe eggs, it is necessary to examine briefly the developmental stages of normally ripe ones.

Stages of Embryonic Development of Rainbow Trout

Stage One - From Fertilization To the Beginning of Cleavage.

Shortly after fertilization takes place, protoplasm begins to form a thin layer over the vitelline matter. Gradually, it concentrates in the animal half of the egg. An hour after 86 fertilization, the embryonic disc emerges a little to the side of the animal pole of the egg. On the third hour after fertilization, fatty drops from the eggs concentrate more densely around the embryonic disc. Until the eighth hour, the size of the embryonic disc varies from 1.3 to 1.9 mm. At that time it becomes round and stands out clearly against the vitelline matter.

Stage Two - Cleavage.

Cell division starts after the tenth hour of fertilization. Eight blastomeres can be observed on the eighteenth hour, and sixteen on the twentieth hour. The size of the embryonic disc during this stage varies from 1.3 to 1.5 mm. From the twenty-first to the twenty-third hour, the number of blastomeres grows from 32 to 64. On the twenty-ninth hour the embryonic disc measures 1.4 mm at the maximum.

Stage Three - Blastomeric and Epithelial Blastula

From the thirtieth hour onwards, the embryonic disc begins to emerge through the surface of the egg. Initially, its

diameter stays the same (up to 1.4 mm), but its height grows to .6 or .7 mm. Small hollow spaces, full of liquid, begin to appear among the blastomeres. This is the stage of the so-called "blastomeric blastula". With more egg cleavage going on, the blastomeres diminish in size and become indiscernible under the microscope. On the fourth day, the embryonic disc becomes flatter, its diameter growing from 1.4 to 1.7 mm. Around the ninety-sixth hour, the embryos pass on to the stage of epithelial blastula. The outer layer of blastomeres becomes denser. The height of the embryonic disc is now .3 mm. On the fifth day, its diameter reaches 1.8 to 1.9 mm.

Stage Four - Formation Of Embryonic Layers

On the sixth day (138-th hour), the embryos enter a new stage of development. The blasto-disc grows to 2.4 or 2.6 mm. The blastomeric layer is very thin in the middle and thicker at the edges.This stage, also called "annular", is the beginning of gastrulation. On the 162-th hour (the seventh day), the diameter of the blasto-disc is between 2.6 and 3.3 mm. The annular stage lasts until the 164-th hour after fertilization. On the 168-th hour, the diamiter of the embryonic disc measures 2.6 to 3.6 mm. A thicker part consisting of blastomeres and called the "end knot" forms at one end of the disc on the 172-nd hour. Its gradual covering with vitelline matter and the formation of embryonic layers begin at this stage.

Stage Five - Formation Of the Embryo's Head and Body

This stage starts with the emergence of the first bodily segments and lasts from the eighth to the twelfth day inclusive.

On the eighth day (186 to 188 hours since fertilization), the embryonic disc has a diameter of 3.6 to 3.8 mm and envelopes about one quarter of the vitelline matter. The embryo looks like a narrow strip, forming the so-called "end tongue" and measuring 1.7 to 2.4 mm in length. On the ninth day, the embryonic disc has a diameter of 4.2 to 4.3 mm, and the embryos are 1.9 to 2.6 mm long. Its covering with vitelline matter from the blasto-disc continues. Even before the blasto-disc has reached the egg's equator, the thick swellings of the eye vesicles emerge at the front end of the embryo. The chorda and the first bodily segments become visible. At the beginning of the day they are only three, growing to as many as 10 at the end of the day. The eye swellings grow fast and turn backwards. A characteristic feature of the /87 formation of the eye vesicles is that their hollowness emerges as a secondary feature. On the tenth day, the blasto-disc reaches the egg's equator. The embryos are 2.9 to 3 mm long. They have nine to ten somites. The eye vesicles are sharply elongated backwards. On the eleventh day, three quarters of the vitelline matter is enveloped by the blasto-disc. The embryos are from 3.4 to 3.6 mm. The number of somites grows fast to nineteen or twenty. Behind the eye vesicles, a dense formation of cells puts the beginning of the auditory vesicles. On the twelfth day, vitelline matter has almost finished its job. The embryos are about 3.6 mm. The bodily somites are 23 or 24. A slight bending appears in the outer layer of the eye vesicles (toward the ectoderm) and a hollow is noticed between the two layers of eye vesicles. The

ectodermal cells, which will form the crystalline lense, begin to divide. Hollow space also appears in the auditory vesicles.

Stage Six - Closing Of the Blastopore and the Emergence Of the Embryos Through the Vitelline Vesicle

This stage begins with the closing of the blastopore and lasts until the emergence of a hemoglobinless blood circulation system (from the thirteenth to the eighteenth day, inclusive). From 4.2 mm at the beginning, the embryos reach 6 mm at the end of the stage. The number of their somites grows from 29 or 30 to 59 or 60. Gradual changes begin to take place in all their organs. On the thirteenth day, the first three brain vesicles begin to be slightly discernible. On the fifteenth and the sixteenth day, the second and third vesicles begin to swell and a fourth vesicle begins to take shape in the elongated brain. On the seventeenth day, a hollowness appears also in the first brain vesicle, which continues to grow on the eighteenth day. The second brain vesicle grows fastest of all and occupies the whole width of the head. The eyes undergo changes, too. On the thirteenth day the lense is linked to the ectoderm in the form of a semi-circle. Gradually, the link becomes thinner, the lense taking a spherical shape and, at the end of the stage, on the eighteenth day, for the first time the lense is completely separated from the ectoderm. At the beginning of the stage, the auditory vesicles are quite far from the eyes (at a distance equal to thirteen somites). Gradually, they grow longer and the distance from them to the closer end of the eyes diminishes

to nine somites. As early as the thirteenth day, a thickening of the epithelium appears at the front end of the head, which represents the beginning of the olfactory placoids. On the fifteenth day, they already emerge as a thickened part of the ectoderm, in the shape of a half-moon, and remain like that until the end of the stage. It is on the fifteenth day that the heart can be observed for the first time, looking like a straight piece of tube. The forming blood vessels show no sign of blood flow. On the fourteenth day the first operculum emerges under the auditory vesicles, and on the eighteenth day, there are already three pairs of them. During this stage, the rudiments of an intestine appear for the first time. The intestine, like the rest of the organs, is solid at the beginning. On the eighteenth day a narrow opening appears at its front end, which gradually moves backwards, without reaching the end of the intestine. The anal aperture is still missing.

Stage Seven - Homoglobinless Blood Circulation

This stage is a comparatively short one: from the nineteenth to the twentieth or twenty-first day. It is characterized by the appearance of blood circulation and some weak heart pulsations. The blood elements are very sparse. Simultaneously, blood islets begin to appear on the surface of the vitalline vesicle. The embryos are now six or seven millimetres long. The number of bodily segments ranges between 59 or 61 to 65 (out of which 40 or 41 are bodily and 22 or 26 are caudal segments). The brain, represented by the three brain vesicles, continues to grow. The hollowness of the first brain vesicle grows larger. The distance between the auditory vesicles and the eyes is equal

to eight or nine somites. By the twentieth day, the olfactory dimples have sunk considerably. A characteristic feature of the stage is the emergence of pectoral fins. On the nineteenth day we see them as a concentration of cells, which, on the following day, take the shape of fins behind the auditory vesicles. The anal aperture opens up and now the intestine is entirely open. The embryos appear to have separated a little at their upper end from the vitelline vesicle.There are already three pairs of opercular arcs.

Stage Eight - Hemoglobin Blood Circulation

This stage covers the twenty-first or second day to the twenty-eight day since fertilization. The embryos measure between seven to eight millimeters. The somites range from 59 to 65, out of which 37 or 38 are bodily ones and 26 to 29 are caudal. Gradually, the embryos' front end separates from the vitelline vesicle up to the opercular arcs. For the first time at the beginning of this stage (on the twenty-second day), in both living and fixed material, a very thin layer of pigment appears in the tapetum nigrum of the eyes. Towards the end of the period (on the twenty-sixth day), the eyes are already strongly pigmented. This stage is known as the "little eyes" stage, since the pigment begins to show clearly through the egg membrane. The embryos are relatively insensitive to mechanical shocks. The eye lense becomes better shaped, with its epithelial cover detaching itself. The distance between the auditory vesicles and the eyes decreases from five or six segments at the beginning,

to three or four at the end of the period. The digestive system also undergoes changes. The liver develops well. The front end of the intestine takes the shape of a stomach with a wider lumen. The heart is divided into an auricle and a ventricle and makes about 84 beats per minute (on the twenty-second day of embryonic development). the liver blood circulation develops gradually with a dense net of blood vessels taking blood out of the liver. The opercular arcs are now four pairs. The embryos become detached at their front end from the vitelline vesicle up to their opercular arcs.

Stage Nine - The Liver-Vitelline Blood Circulation System

This period covers the twenty-ninth to the thirty-third day since fertilization. The embryos measure 10 to 11 mm in length. The somites number 63 to 65, out of which 37 or 38 are bodily and 26 or 27 are caudal ones, meaning that the caudal segments have decreased in comparison with the previous stage. The eyes are strongly pigmented. The auditory vesicles are found very close behind the eyes, the distance to them being smaller than two bodily segments. For the first time, otoliths appear in them. The stomach is better differentiated. Yellow pigment appears in the intestine from the bile which flows into it. The intestine-vitelline vein stops functioning and the blood passes entirely through the liver, which is already well developed. Blood vessels appear in the head and the branchiae, which means that the branchial blood circulation has emerged. The four pairs of branchial arcs are well developed. The operculum grows

gradually. On the thirtieth day, it covers the first pair of branchial arcs. The dorsal fin begins taking shape on the thirty-first day. he pectoral fins have become rounded. More seldom at the beginning of the period, and more and more often later on in it, tiny brown dots, the "hatching glands"[1], can be observed appearing on the pericardium and the head. /89

Stage Ten - Differentiation Of Upper and Lower Cones Of Bodily Musculature

This stage covers the thirty-fourth to the thirty-seventh day. The embryos grow from 11.5 to 14.5 mm. The number of bodily segments does not change. As early as the thirty-fourth day, melanophores appear in the upper side of the body and around the intestine. Their number grows gradually and on the thirty-seventh day pigment cells spread to the head, too. The "hatching glands" are already well developed and show clearly on the living specimens. They spread ever more densely. The operculum grows still more, with the last two pairs of branchiae remaining uncovered. The blood vessel network on the vitelline vesicle reaches its highest development. Blood capillaries develop in the pectoral and caudal fins.

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[1]According to Dissler (1957), in the case of Onchorynchus keta and O. gorbusha varieties, the "hatching glands" appear during the eleventh stage, and, according to Vassilev (1959), they appear during the tenth stage of the embryos' development.

Stage Eleven - The formation Of the Mouth Opening

On the forty-first of forty-second day, the abdominal fins become visible like tiny growths behind the vitelline vesicle. The mouth opening takes shape, but the jaw is still immobile. During this stage, hatching begins. In the course of our experiments, the first fish was hatched on the forty-second day (338.8 degree-day). Mass hatching began on the day after (342.6 degree-day). Newly hatched fishes measure 15 to 17 mm in length. Their abdominal and their dorsal fins are better shaped. Their lower jaw is mobile. They make fast breathing motions with their jaws and pectoral fins. They show a great number of melanophores on their heads. The upper part of the body has also acquired well developed pigment cells. Mesenchyme tissue, from which fin rays will develop, begins to accumulate at the base of the pectoral, caudal and anal fins. The body muscles are in the form of double cones. The intestine is strongly pigmented by the bile. The heart throbs with 114 to 116 beats per minute. At hatching time, the operculum does not cover yet the last two pairs of branchial arcs. The branchial stamens, that have developed on the last branchial arcs, later appear on the rest of the branchiae (on the fiftieth day since fertilization).

Development Of Embryos From Overripe Eggs

a. Development From Seven Days Overripe Eggs

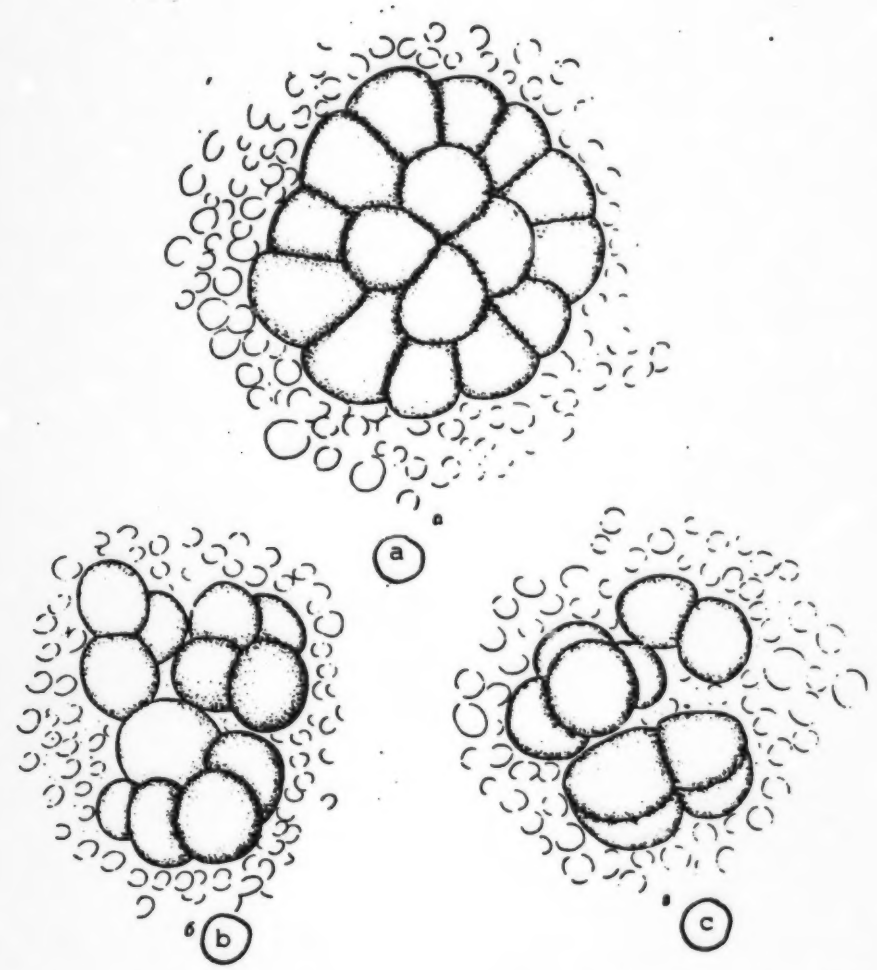
The changes which the eggs undergo immediately after fertilization are similar to those of the control group. Cleavage begins before the fifteenth hour at a water temperature of 6 ^{0}C

and finishes before the forty-first hour. In general, it proceeds in a normal manner. The embryonic disc measures from .9 to 1.2 mm. In single instances, uneven cleavage and irregular arrangements of blastomeres are observed at the early stages of development (Fig.1b).

Table 1 presents a comparison between the rate of growth of the experimental and control embryos, as well as between the average temperatures and degree-days of the different embryonic stages.

The third and fourth stages of embryonic development run /90 almost simultaneously with those of the control group. A delay was only noticed during gastrulation, which, in the case of the control fishes started a day earlier, i.e. on the fifth day. During the fifth stage, when the head and body are formed, some abnormally developed embryos were observed, having curved front parts of the body and a reduced number of bodily segments. The embryos' length at the beginning of the stage is 1.4 mm. Immediately before the blastopore is closed (on the thirteenth day), the bodily somites are 16 to 20. The organ development of the experimental embryos does not differ from that of the control fishes.

During the sixth stage, after the blastopore is closed, the embryos are 4.2 to 4.3 mm long. The number of bodily segments is 22 to 27 at the beginning, and 46 at the end of the period. A number of embryos are rather anaemic and shorter in length than the control ones. In terms of their eye development and number of somites, the experimental embryos are one day behind the /93 control ones.



Фиг 1. Зародиши на стадий 16 бластомера от дъговата пъстърва *(Salmo irideus* Gibb.)
2×8

а — контрола, нормално развитие. 20 часа от оплождането; *б* — неправилно дробене при 7 дни презрял хайвер. 24 часа от оплождането; *в* —неправилно дробене при 14 дни презрял хайвер. 30 часа от оплождането. Бластомерите лежат в отделни групи и са с различни размери.

Fig.1.Embryos at the stage of 16 blastomeres of rainbow trout (Salmo irideus gibb.)
2X8

a Controls, normal development, 20 hours after fertilization

b Irregular cleavage of 7 days overripe eggs, 24 hours after fertilization

c Irregular cleavage of 14 days overripe eggs, 30 hours after fertilization. The blastomeres are arranged in different groups and vary in size.

Таблица 1 Tabelle 1

Сравнение между етапите от ембрионалното развитие на опитни и контролни зародиши от *Salmo irideus Gibb.* Опит, проведен през април—май 1965 г.
Vergleich zwischen den Etappen der embryonalen Entwicklung der Versuchs- und Kontrollembryone von *S. irideus*. Versuch, der im April—Mai 1965 durchgeführt wurde

(1) Етапи Etappen	Контрола Kontrolle (Control)			7 дни презрял хайвер 7-tägiger überreifer Laich (7 day overripe)			14 дни презрял хайвер 14-tägiger überreifer Laich (14 day overripe)		
	часове (дни) Stunden (Tage) (hrs days)	средна температура за етапа Mittel t° für die Etappe (°C)	градусодни Tagesgrade (d°)	часове (дни) Stunden (Tage) (hrs days)	средна температура за етапа Mittelwerte für die Etappe (°C)	градусодни Tagesgrade (d°)	часове (дни) Stunden (Tage) (hrs days)	средна температура за етапа Mittelwerte für die Etappe (°C)	градусодни Tagesgrade (d°)
1	2	3	4	5	6	7	8	9	10
I и II	до 29-ия час bis zur 29 Stunde (2)	7°	до 14 (3)	до 41-ия час bis zur 41 Stunde (4)	6,9°	до 20,8 (5)	до 46-ия час bis zur 46 Stunde (6)	6,9°	20,8
III	от 30-ия час до 5-ия ден вкл. Von der 30. Stunde bis zum 5. Tag einschliesslich (7)	8°	22—46	от 43-ия час до 5-ия ден вкл. Von der 43. Stunde bis zum 5. Tag einschliesslich (8)	7,2°	28—42,5	от 47-ия час до 5-ия ден вкл. Von der 47. Stunde bis zum 5. Tag einschliesslich (9)	7°	27,4—41,8
IV	от 6-ия до 8-ия ден Vom 6. bis zum 8. Tag (10)	7,8°	54—69	от 6-ия до 8-ия ден Vom 6. bis zum 8. Tag (10)	7°	50,1—63,7	от 6-ия до 8-ия ден Vom 6. bis zum 8. Tag (10)	7,5°	49,4—64,3
V	от 8-ия до 12-ия ден Vom 8. bis zum 12. Tag (11)	6,7°	75,8—95,6	от 9-ия до 13-ия ден Vom 9. bis zum 13. Tag (12)	7,2°	70,9—99,5	от 9-ия до 13-ия ден Vom 9. bis zum 13. Tag (12)	8,9	72,8—108,5
VI	от 13-ия до 18-ия ден Vom 13. bis zum 18. Tag (13)	6,7°	102,8—135,7	от 14-ия до 18-ия ден Vom 14. bis zum 18. Tag (14)	8,2°	107,1—140,7	от 14-ия до 16-ия ден Vom 14. bis zum 16. Tag (15)	8,8°	117,5—134,9

Table 1. Comparison between the stages of embryonic development of experimental and control embryos of Salmo irideus Gibb. The experiment was carried out in April and May of 1965

1 Stages
2 Up to the 29th hour
3 Up to 14
4 Up to the 41st hour
5 up to 20.8
6 Up to the 46th hour
7 From the 30th hour to the 5th day incl.
8 From the 43rd hour to the 5th day incl.
9 From the 47th hour to the 5th day incl.
10 From the 6th to the 8th day
11 From the 8th to the 12th day
12 From the 9th to the 13th day
13 From the 13th to the 18th day
14 From the 14th to the 18th day
15 From the 14th to the 16th day

Таблица 1 (продължение)

1	2	3	4	5	6	7	8	9	10
VII	(1) от 19-ия до 21-ия ден Vom 19. bis zum 21. Tag	6,9°	142,3—156,5	(2) от 19-ия до 22-ия ден Vom 19. bis zum 22. Tag	8,8°	149,2—176,1	(3) от 17-ия до 19-ия ден Vom 17. bis zum 19. Tag	9,5°	145,1—163,5
VIII	(4) от 21—22 до 28-ия ден Vom 21—22. bis zum 28. Tag	7,9°	163,5—212,0	(5) от 23-ия до 26-ия ден Vom 23. bis zum 26. Tag	8,4°	184,1—209,8	(6) от 20-ия до 23-ия ден Vom 20. bis zum 23. Tag	8,6°	171,8—197,8
IX	(7) от 29-ия до 33-ия ден Vom 29. bis zum 33. Tag	8,5°	220,7—254,4	(8) от 27-ия до 29-ия ден Vom 27. bis zum 29. Tag	8,5°	218,0—235,3	(9) от 24-ия до 28-ия ден Vom 24. bis zum 28. Tag	8,8°	206,8—241,9
X	(10) от 34-ия до 37-ия ден Vom 34. bis zum 37. Tag	8,5°	262,7—289,7	(11) от 30-ия до 35—36-ия ден Vom 30. bis zum 35—36. Tag	8,8°	244,0—288,2	(12) от 29-ия до 33-ия ден Vom 29. bis zum 33. Tag.	8,8°	250,7—285,8
XI	(13) от 38-ия ден Vom 38 Tag	над 8,7° Über 8,7°	от 298,7	(14) от 37-ия ден Vom 37 Tag	(15) над 8,7° Über 8,7°	от 297,0	(16) от 34-ия ден Vom 34. Tag	над 9,2° Über 9,2°	(17) от 295,8
Начало на излюпване Anfang des Ausschlüpfens (start of hatching)	(18) от 43-ия ден Vom 43. Tag		342,6	(19) от 41-ия ден Vom 41. Tag		341,6	(20) от 39-ия ден Vom 39. Tag		342,5

Table 1 (continuation)

1 From the 19th to the 21st day
2 From the 19th to the 22nd day
3 From the 17th to the 19th day
4 From the 21-22 day to the 28th day
5 From the 23rd day to the 26th day
6 From the 20th to the 23rd day
7 From the 29th to the 33rd day
8 From the 27th to the 29th day
9 From the 24th to the 28th day
10 From the 34th to the 37th day
11 From the 30th to the 35-36th
12 From the 29th to the 33rd day
13 From the 38th day
14 From the 37th day
15 Over 8.7°
16 From the 34th day
17 From 295.8
18 From the 43rd day
19 From the 41st day
20 From the 39th day

The beginning of the seventh stage coincides, in terms of days and degree-days, with that of the control fishes of April 28. The embryos are 5 to 5.2 mm long at the beginning of the period.The number of bodily segments ranges from 49 to 65. Similarly to the previous stage, there are abnormal changes in the embryos, such as a curved spinal cord, some of them looking very weak and transparent.

The appearance of hemoglobin in the blood, characterizing the beginning of the eighth stage, is two days (twenty degree-days) later than in the control group. The eye pigment comes later, too. The embryos, shorter in length and with curved chordas, measure 4.4 mm, as opposed to the 6.6 mm of the control ones.

During the ninth stage, a number of the embryos still have very little pigment in their eyes, whereas, at the same time, the control embryos' eyes are strongly pigmented. Some of the embryos show curved chordas. The rest, which, outwardly, look normal, are smaller in size and paler in colour than the control ones. The number of bodily segments is about 59 or 60, with 63 to 65 in the control embryos.

During the tenth stage, some of the embryos have small eyes (microphthalmia) or a curved spinal cord. In the live specimens the "hatching glands" can be clearly distinguished.

The eleventh stage begins on the thirty-seventh day. At its beginning the embryos are 11 to 13 mm long, and 13 to 14 mm at the time of hatching. The first fish was hatched on the forty-first day after fertilization (341.6 degree-days), similar to

the hatching time of the control fishes. The heart throbs with 92 to 120 beats per minute. On the seventh day after hatching, the embryos are 15 to 18 mm long. The muscular segments begin growing over the vitelline vesicle. Faint rays begin to emerge at the base of the dorsal and caudal fins.

b. Development From Fourteen Days Overripe Eggs

In general, embryos developed from fourteen days overripe eggs show greater aberrations in their development. During cleavage, at the eight- and sixteen-cell stages already, an irregular arrangement of blastomeres can be observed. In the sample from the eight-cell stage, forty-four percent of the material show irregular cleavage. At the sixteen-cell stage, blastomeres are more frequently found to be arranged in separate groups or in a disorderly manner (Fig.1c).

During the third and fourth stages, there are no visible aberrations in the embryos' development.

The fifth stage develops simultaneously with that of the other experimental fishes and the control ones. Even before the blastodisc reaches the equator, a curvature appears in the front part of some of the embryos. By the end of the stage, in ever greater number of the embryos can be observed a twisted spinal cord. The embryos are frail and weak. The number of somites before the closing of the blastopore is about 24. During the sixth stage, the embryos are 4.8 to 6 mm long. The aberrations observed in this period are similar to the ones in the previous stage.

The stage of hemoglobinless blood circulation for the experimental embryos is a comparatively short one (two days), which is most likely due to the higher average temperature (9.5^{0}C). The embryos look very weak and twisted. They are about 6 mm long. /94

Hemoglobin appears in the blood on the twentieth day (during the eighth stage). During that time more than fifty percent of the fixed embryos show aberrations in their development. The most frequent abnormalities are shorter bodies, twisted chordas and microphthalmia. The vitelline vesicle is soft and pale. When attempting to isolate the embryos from the egg membrane, the vitelline vesicle bursts open very easily and its contents run out. The eyes are only slightly pigmented and their pigment does not show through the egg membrane. Only at the beginning of the next stage, does a macroscopic examination of the eggs reveal pigmented eyes, and not in all embryos, at that. At the end of the eighth stage, the embryos measure 4.3 to 5.5 mm in length. The heart pulsates with about 70 beats per minute.

During the ninth stage about 40% of the embryos isolated from their egg membranes show a twisted spinal cord. At the end of the period they reach 8.3 to 8.6 mm in length. It is only on the twenty-eighth day that the vitelline vesicle can be easily isolated without being torn, due to the denser network of blood vessels covering it. At that time the operculum does not cover any of the branchial arcs, while in the controlled situation the first pair of branchial arcs has already been covered.

During the tenth stage there can still be found under-developed embryos with poorly pigmented eyes, whereas in the control group the pigment appears in the eyes as early as the eighth stage. The embryos' length is 8 to 13.3 mm, but there are still some which measure 6.6 mm. Until the end of this period the prevailing number of embryos are 10 mm long. Early in the stage, bile pigment appears in the intestine, characteristic of the ninth stage for the control embryos. The mouth opening also takes shape. The operculum covers only the first pair of branchial arcs. Out of 30 embryos, 10 have twisted spines, poorly pigmented eyes or microphthalmia. Despite the delay in the embryos' general development, the "hatching glands" develop well and can be observed in live specimens. A number of embryos have a very soft vitelline vesicle, which bursts easily when they are being isolated. The heart pulsates with 106 to 120 beats per minute. The capillary vessels develop well in the head, and, by the end of this period (the thirty-third day), in the caudal fin as well. The tenth stage of normally developed embryos is characteristic with the emergence of melanophores on the body. In the case of the embryos of 14 days overripe eggs, though, the pigment is very poorly developed and many of the embryos are colourless having no pigment cells whatsoever.

During the eleventh stage the embryos are 10 to 15 mm long. The prevailing number of embryos measure 12 or 13 mm in length on the second day after hatching. The first fish was hatched on the thirty-ninth day after fertilization (342.5 degree-days). At this time the operculum covers the first two

pairs of branchial arcs. Branchial stamens first develop on the last two pairs of branchial arcs. Mesenchymal tissue builds up at the base of the double and single fins. Even after hatching (on the first and second days), some of the embryos have very poorly developed pigment on their bodies. Others' blood is colourless, with sparse erythrocytes. The eyes are tiny.
A large number of the experimental embryos died in the eggs before hatching. A histological examination of the condition of those embryos' "hatching glands" will be carried out at a later date.

Mortality Rate and Fertilization Of Overripe Eggs /95

Table 2 presents data on the mortality rate of the embryos in our 1964 experiments. During the 1965 experiments, the mortality rate was not registered, because a large number of embryos were fixed on a daily basis. Percentages are calculated based on the total number of experimental eggs, including the unfertilized ones. The unfertilized eggs were counted, when pigment appeared in the embryos' eyes.

Table 2 shows that the eggs' mortality is determined to a great extent by the producing mothers. While in the case of fishes No.1, 2, and 3 the mortality of normally ripe eggs is comparatively high, in the case of fishes No.4, 5, and 6 it is considerably lower (.8 to 5.46). When the eggs from the same fishes become four days overripe, though, their mortality rate rises for each one of them accordingly. As the table shows, the lowest mortality rate for all three samples was observed in the

% mortality

Таблица 2 Tabelle 2

Смъртност в проценти на зародишите от нормално зрял, 4 и 8 дни презрял хайвер. Опит, проведен през май—юни 1964 г.
Sterblichkeitsprozente der Embryone von normal reifem, 4 und 8 tägigen überreifen Laich. Versuch, im Mai—Juni 1965 durchgeführt

8 days overripe

Развитие в дни Entwicklung in Tagen (1) / Риба № Fisch N°	normal-ripe Нормално зрял хайвер Normal reifer Laich						4 days overripe 4 дни презрял 4 tägiger überreifer Laich			8 дни презрял 8 täg. überreifer Laich
	1	2	3	4	5	6	4	5	6	6
(2) От 1 до 10-ия ден 1. bis 10. Tag days	0,62	1,06	1,27	0,75	1,48	—	0,99	1,32	3,67	0,55
От 11 до 20-ия ден 11. bis 20. Tag (3)	0,62	3,20	0,63	0,75	—	—	2,47	0,42	0,67	0,55
От 21 до 30-ия ден 21. bis 30. Tag (4)	3,10	2,84	0,31	—	1,99	0,80	6,42	7,16	0,34	0,27
От 31-ия ден до излюпването Vom 31. Tag bis zum Ausschlüpfen (5)	9,00	5,33	11,78	0,75	1,99	—	2,47	—	—	—
Всичко до началото на излюпването Alles bis zum Anfang des Ausschlüpfens (6)	13,34	12,43	12,99	2,25	5,46	0,80	12,35	8,90	4,68	1,37
Неоплоден хайвер в % спрямо общия брой хайверени зърна Nicht befruchteter Laich in % im Vergleich zur Gesamtzahl der Laichkörner. (7)	14,90	12,10	17,19	3,78	13,93	4,38	3,61	13,0	0,66	10,13

Table 2. Percentages of mortality of embryos from normally ripe, 4 and 8 days overripe eggs. The experiment was carried out in May and June, 1964

1 Development in days
2 From the 1st to the 10th day
3 From the 11th to the 20th day
4 From the 21st to the 30th day
5 From the 31st day until hatching
6 Total prior to hatching
7 Unfertilized eggs in % of total number of eggs

embryos developed from the eggs of fish No.6. A similar relation can be also observed with respect to the percentage of unfertilized eggs. It is interesting to note that the lowest percentage of unfertilized eggs was again observed in the case of fish No.6 (.66), when they were 4 days overripe. It can be /96 assumed that the eggs had been in their best state for fertilization at the time of the second removal, and not the first one. These assumptions, however, can be proved only after a thorough cytological examination of the samples of overripe eggs.

Hatching Of the Embryos

The beginning of hatching was recorded for all samples in both 1964 and 1965. The mass hatching, which usually occurs a few days later, and the completion of the hatching process were recorded only in 1964. Table 3 presents the results of those observations.

The table demonstrates that the number of days and degree-days before the beginning of hatching decreases gradually. The speeding up of development depends on the higher water temperatures at the end of the incubation period. The first ones to hatch were the embryos produced from 8 days overripe eggs (in 30 days or 331 degree-days). The mass hatching[1] of embryos from normally ripe eggs occurred 4 to 6 days after the appearance of the first fishes. It was only in the case of fish No.3, that /97 an interval of 13 days elapsed between the beginning of hatching

[1] The day when over 50% of the embryos have left the egg membrane is considered the mass hatching day.

Таблица 3 Tabelle 3

Продължителност на развитието в дни и градусодни до излюпване на зародишите Опит, проведен през май—юни 1964 г.
Entwicklungsdauer in Tagen und Tagesgraden bis zum Ausschlüpfen der Embryone. Versuch, im Mai—Juni 1964 durchgeführt

Степен на зрелост на хайвера Reifegrad des Laichs	Риба № Fisch N°	Начало на излюпването дни градусодни Beginn des Ausschlüpfens Start hatch		Масово излюпване дни градусодни Massenhaftes Ausschlüpfen median hatch		Край на излюпването дни градусодни Ende des Ausschlüpfens end hatch	
ripeness		Tage days	Tagesgrade d°	Tage days	Tagesgrade d°	Tage days	Tagesgrade d°
Нормално зрял хайвер	1	38	371,8	—	—	—	—
	2	38	371,8	—	—	—	—
	3	38	371,8	51	525,6	—	—
Normal reifer Laich (normal ripe)	4	34	353,6	39	413,2	—	—
	5	34	353,6	38	400,8	44	473,8
	6	34	353,6	40	425,8	43	461,8
4 дни презрял хайвер	4	33	356,6	41	453,0	—	—
	5	31	332,8	39	429,6	—	—
	6	33	356,6	35	381,0	39	429,6
4-tägiger überreifer Laich (4 days overripe)							
8 дни презрял хайвер	6	30	331,0	35	392,0	37	415,4
8-tägiger überreifer Laich (8 days overripe)							

Table 3. Embryos' development in days and degree-days before hatching. The experiment was carried out in May and June, 1964

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and the mass hatching day. The same fish produced a total of 30% unfertilized and dead eggs. For the embryos hatched from 4 days overripe eggs (from fishes No.4 and 5), the interval between the beginning and the mass hatching day was 8 days. The data on the hatching of the eggs of fish No.6 seems to contradict that of the other fishes. While in the case of the eggs removed the first time the period between the beginning of hatching and the mass hatching day is 6 days (72.2 degree-days), in the case of 4 days overripe eggs it covers 2 days (24.4 degree-days), and in the case of 8 days overripe eggs, the interval increases again to 5 days (61.0 degree-days). These results once again support the assumption that when the eggs were removed from fish No.6 the first time, they had been underripe and that it was four days later that they were at their best moment for fertilization and development.

During the 1965 experiments, the beginning of hatching of normally ripe eggs for all five mothers began on the forty-third day (342.6 degree-days). In the case of 7 days overripe eggs, it began on the forty-first day (341.6 degree-days) and in the case of 14 days overripe eggs, it started on the thirty-ninth day (342.5 degree-days), which means that in all three cases the number of degree-days measuring the interval prior to hatching is practically equal (Table 1).

Discussion Of Results

Summarizing the results of our experiments with overripe eggs of rainbow trout, we must point out that the longer stay of eggs in the fish's body after the normal time of ovulation, has, in general, an unfavourable effect on the embryos' develop-

ment. Twisted chordas, delayed pigmentation of the eyes and slower growth are some of the most frequent aberrations noticed in the affected embryos. In the case of embryos from 14 days overripe eggs, it can be observed that as late as the tenth stage, some of them still have poorly pigmented eyes, whereas in the normal embryos eye pigment appears early in the eighth stage. Pigment cells appeared on some of the experimental embryos' bodies only after hatching, while in the control embryos this happened early in the tenth stage. The aberrations observed in the development of embryos from 14 days overripe eggs are very similar to those described by Ostroumova (1963) in her experiments with rainbow trout embryos in water with a low oxygen content (5 to 7 ml/l of water). The author observed a considerable delay in the experimental embryos' growth and development. A large number of the enbryos were deformed and had rudimentary eyes and twisted spinal cords. The rate of formation of bodily somites was also delayed. The blood circulation system of the experimental embryos was poorly developed and showed numerous anomalies. We, too, noticed a delayed appearance of hemoglobin in the blood of the experimental embryos. According to data from the same author, (Ostroumova, 1962), the first signs of the formation of hemoglobin in the blood of the rainbow trout embryos were recorded at the moment when the rudiments of pectoral fins appeared (proved with benzidine tests). When the water temperature was 8^{o}or 9^{o}C, hemoglobin appeared on the thirteenth day. The embryos had 47 or 48 bodily segments. As

early as the twelfth day, the first weak heart beats were recorded. Erythrocyte movement in the blood current was registered only on the fourteenth or fifteenth day. Our observations of normally developing embryos established hemoglobin in the embryos' blood only on the twenty-first or the twenty-second day (163 degree-days). The bodily segments at this stage were 59 to 61. The rudiments of pectoral fins appeared during the seventh stage of "hemoglobinless" blood circulation after about 140 degree-days. We would like to suggest that the stage of "hemoglobinless" blood circulation is an arbitrary one, because, in fact, there is some hemoglobin in the blood even before that but in such small quantities that they cannot be observed in live specimens. Therefore, physiologically, the two stages represnt one period which is divided only on the basis of outside observation.

To what extent are overripe eggs capable of fertilization? It can be concluded from our experiments that the percentage of unfertilized eggs in normally ripe and 4 days overripe eggs, produced by the same mother, is more or less equal. What proved to be of greater importance in our experiments are the individual characteristics of the various producing mothers, their age and the quality of their eggs, all of which factors should be taken into cosideration in fish farming. Kharchenko (1949, 1957) established 100% fertilization of 7, 14 and 16 days overripe salmon eggs and of 15 days overripe eggs of Coregonus albula. A lower percentage of fertilization was recorded for underripe eggs (69 and 64 % accordingly). The conclusion which can be drawn from these experiments is that overripe eggs preserve their

fertilizing capacity for a long time. Nussenbaum (1949), however, established for M. fossilis that the eggs' fertilizing capacity decreases if they remain too long in the mother's ovaries. he increase in temperature had a particularly detrimental effect on them. All these authors do not take into account the individual differences between the producing mothers, which, undoubtedly, affect the quality of the eggs and their capacity for fertilization.

With respect to the mortality rate, in our experiments with 4 days overripe eggs we observed a 3.44 to 10.10% increase in mortality compared to the death rate of normally ripe eggs produced by the same mothers. Mrsik (1923) has developed a table showing that the embryos' mortality prior to hatching increases gradually from 3% for normally ripe eggs to 6% for 4 days overripe, 8% for 7 days overripe, 30% for 13 days overripe and 54% for 21 days overripe eggs. Privolnev, Kharchenko (1940) wrote that salmon embryos hatched from overripe eggs show a higher mortality rate and that overripe eggs are less resistant to external influence. These observations concern only the first days after fertilization. Kharchenko (1949) established a higher mortality rate for overripe salmon eggs. While the mortality rate of normally ripe eggs is 5.8%, that of overripe eggs is 15.8%. Throughout his experiments, the mortality of embryos hatched from overripe eggs continued to be 27% higher than that of the control fishes.

In these terms, the data obtained during our experiments is very much in line with the information provided by other authors. The overripeness of eggs affects adversely the embryos'

viability, and the increase in overripeness also causes an increase in their mortality rate.

Conclusion

/99

Several general conclusions can be drawn from the experiments with 4 and 8 days overripe eggs (carried out in 1964) and with 7 and 14 days overripe eggs (1965).

The overripeness of eggs has a detrimental effect on the embryos' development. Fourteen days overripeness proved to have a particularly harmful influence, causing considerable aberrations in the embryos. The most frequent aberrations are a twisted spinal cord, a delayed development of the blood circulation system and the appearance of hemoglobin in the blood, delayed pigmentation of the eyes and the body, and slowed down growth.

The fertilizing capacity of 4 days overripe eggs is not reduced. The rate of fertilization varies even for producers of normally ripe eggs, depending on their characteristics.

The mortality rate of 4 days overripe eggs increases from 3.44% for normally ripe eggs to 10.10%.

The time of development prior to hatching (in degree-days) is almost equal for both the control and the experimental embryos. In 1964 the shortest incubation period was recorded for the 8 days overripe eggs, owing to the higher water temperature. Overripeness affects the rate of development at different stages, the final hatching time for both control and experimental embryos levelling out.

The two stages of blood circulation with and without hemoglobin (Dissler, 1954 and 1957) can be considered as one physiological period, because hemoglobin appears in the blood a lot earlier than recorded (Ostroumova, 1962).

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